Adenosine and Prostaglandin E$_2$ Cooperate in the Suppression of Immune Responses Mediated by Adaptive Regulatory T Cells*

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Adaptive regulatory T cells (Tr1) are induced in the periphery upon encountering cognate antigens. In cancer, their frequency is increased; however, Tr1-mediated suppression mechanisms are not yet defined. Here, we evaluate the simultaneous involvement of ectonucleotidases (CD39/CD73) and cyclooxygenase 2 (COX-2) in Tr1-mediated suppression. Human Tr1 cells were generated from peripheral blood mononuclear cell-derived, sorted CD4$^+$CD25$^-$ T cells and incubated with autologous immature dendritic cells, irradiated COX-2$^+$ or COX-2$^-$ tumor cells, and IL-2, IL-10, and IL-15 (each at 10–15 IU/ml) for 10 days as described (Bergmann, C., Strauss, L., Zeidler, R., Lang, S., and Whiteside, T. L. (2007) *Cancer Immunol. Immunother.* 56, 1429–1442). Tr1 were phenotyped by multicolor flow cytometry, and suppression of proliferating responder cells was assessed in carboxyfluorescein diacetate succinimidyl ester-based assays. ATP hydrolysis was measured using a luciferase detection assay, and levels of adenosine or prostaglandin E$_2$ (PGE$_2$) in cell supernatants were analyzed by mass spectrometry or ELISA, respectively. Intracellular cAMP levels were measured by enzyme immunoassay. The COX-2$^+$ tumor induced a greater number of Tr1 than COX-2$^-$ tumor ($p < 0.05$). Tr1 induced by COX-2$^+$ tumor were more suppressive, hydrolyzed more exogenous ATP ($p < 0.05$), and produced higher levels of adenosine and PGE$_2$ ($p < 0.05$) than Tr1 induced by COX-2$^-$ tumor. Inhibitors of ectonucleotidase activity, A$_{2A}$ and EP$_2$ receptor antagonists, or an inhibitor of the PKA type I decreased Tr1-mediated suppression ($p < 0.05$), whereas rolipram, a PDE$_4$ inhibitor, increased the intracellular cAMP level in responder cells and their susceptibility to Tr1-mediated suppression. Tr1 present in tumors or the peripheral blood of head and neck squamous cell carcinoma patients co-expressed COX-2, CD39, and CD73. A concomitant inhibition of PGE$_2$ and adenosine via the common intracellular cAMP pathway might be a novel approach for improving results of immune therapies for cancer.

Human malignancies potentiate their own progression and survival using various endogenous molecules to create the microenvironment supportive of tumor growth. The accumulation of oncogenic mutations and the development of new enzymatic activities enable the tumor to produce a multiplicity of factors, which play an important role in its escape from the host immune system. One of these factors is prostaglandin E$_2$ (PGE$_2$),[2] a major product of cyclooxygenase 2 (COX-2) activity. COX-2 is overexpressed by many human malignancies, and its expression has been linked to tumor progression and poor patient survival (1–3). Adenosine is another endogenous factor with anti-inflammatory properties that is generated through the activity of ectonucleotidases, CD39 and CD73, expressed on various cell types, including regulatory T cells (Treg) (4–6) as well as tumor cells (7). The biologic importance of ectonucleotidases activity is supported by a recent finding that CD73 expression on tumor cells promotes tumor growth and formation of metastasis (7). Adenosine is one of the major immunosuppressive factors utilized by Treg for reducing responses to self, regulating tolerance to tissue grafts or cancer, and preventing autoimmune diseases (4, 8, 9). Recent findings suggest that CD4$^+$CD25$^+$FOXP3$^+$CD39$^+$ Treg play an important role in the pathogenesis of multiple sclerosis because they fail to control IL-17-mediated autoimmune inflammation (9). The frequency and functions of Treg are elevated in the peripheral blood and at tumor sites of cancer patients (10, 11) but are decreased in patients with autoimmune disorders (12).

In humans, at least two types of Treg exist: (*a*) naturally occurring Treg (nTreg), which develop in the thymus and mediate suppression by cell contact-dependent mechanisms involving Fas/Fas ligand or granzyme B/perforin pathways (13–15), and (*b*) adaptive Treg (Tr1), which arise in the periphery upon antigen exposure and suppress effector T cells (responder

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[2] The abbreviations used are: PGE$_2$, prostaglandin E$_2$; PBMC, peripheral blood mononuclear cell(s); RC, responder cell(s); CFSE, carboxyfluorescein diacetate succinimidyl ester; PDE, phosphodiesterase; HNSCC, head and neck squamous cell carcinoma; COX, cyclooxygenase; Treg, regulatory T cell(s); nTreg, naturally occurring Treg; Tr1, adaptive Treg; IVA, *in vitro* activated; R, receptor(s); NC, healthy donors; AD, active disease; NED, no evidence of disease; mAb, monoclonal antibody; PE, phycoerythrin; Ab, antibody; S, suppressor; (R_p)-8-Br-cAMPS, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, R_p isomer; α,β-methylene ADP, adenosine 5′-(α,β-methylene)diphosphate; MACS, magnetically activated cell sorting; GITR, glucocorticoid-induced TNF receptor.

cells (RC)) mainly by cell contact-independent mechanisms such as IL-10 and/or TGF-β production (8, 16, 17). It has been reported that in addition to IL-10 and TGF-β Tr1 also express COX-2 and produce immunosuppressive PGE$_2$ (18). However, it has not been determined whether suppression mediated by Tr1 is also dependent on the adenosinergic pathway. We have recently reported that nTreg express ectonucleotidases and generate adenosine to mediate suppression. It was, therefore, important to determine whether suppression mediated by Tr1 cells is also dependent on adenosine production.

Adenosine binds to A$_1$, A$_{2a}$, A$_{2b}$, and A$_3$ receptors (R), which are expressed on the surface of various cell types. In immune cells, suppressive effects of adenosine are largely mediated through A$_{2a}$R signaling with a concomitant up-regulation of intracellular cAMP (19, 20). PGE$_2$ mediates its biological effects through EP$_1$, EP$_2$, EP$_3$, and EP$_4$ receptors (21). Stimulation via the EP$_2$R and EP$_4$R leads to an intracellular increase and activation of cAMP (22) with a concomitant decrease in immune cell proliferation and other functions. The existence of links between the prostanoid and adenosinergic transcellular signaling pathways has been emphasized in the literature (19–23).

This study is the first to report ectonucleotidase expression on human Tr1 cells and the ability of Tr1 cells to generate adenosine *ex vivo* in the presence of COX-2$^+$ or COX-2$^-$ tumor cells. The hypothesis tested is that adenosine and PGE$_2$ produced by human Tr1 have additive effects in down-regulating functions of immune cells through intracellular cAMP elevation. Although immunosuppression associated with elevated cAMP levels has been reported previously (24, 25), our data show it is a key component of Treg-mediated suppression (26). In addition, we tested the hypothesis that Tr1 cells generate adenosine and PGE$_2$ in the tumor microenvironment and peripheral blood of cancer patients. Because both molecules use the same intracellular signaling pathway, their cooperation is likely to contribute to Tr1-mediated suppression of antitumor immune responses.

MATERIALS AND METHODS

Tumor Cell Lines—PCI-13, a COX-2$^+$ HNSCC tumor cell line, was established from a primary tumor and maintained in our laboratory as described previously (27). A COX-2$^-$ HNSCC cell line, ANT-1, was established at the University of Munich, Munich, Germany and was a gift from Dr. R. Zeidler. We used the PCI-13 cell line in previous loss of COX-2 function studies, whereas ANT-1 was used in gain of COX-2 function experiments (28). The cell lines were cultured in RPMI 1640 medium supplemented with 10% ΔFCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/liter L-glutamine at 37 °C. All cell lines were routinely tested and found to be *Mycoplasma*-free.

Collection of Peripheral Blood Mononuclear Cells—Blood samples (20–30 ml) were obtained from 15 healthy donors (NC) and 10 patients with HNSCC. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. We obtained peripheral blood from five HNSCC patients with active disease (AD) and five patients who were disease-free (NED) after systemic oncologi-

cal treatment. These patients were seen in the Outpatient Clinic of the Department of Otolaryngology at the University of Pittsburgh Medical Center. Patients with AD donated blood prior to surgery. Patients with NED were treated with surgery plus radiochemotherapy, and all completed therapy 6–12 months prior to phlebotomy for this study. Blood was drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients (GE Healthcare). PBMC were recovered, washed in AIM V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

IVA—Tr1 were generated from PBMC using the previously described *in vitro* system (IVA) (29). Briefly, monocytes were separated from the lymphocyte fraction via plastic adherence and cultured in AIM V medium supplemented with GM-CSF (1,000 IU/ml) and IL-4 (4 ng/ml) for 7 days to generate immature dendritic cells. CD4$^+$CD25$^-$ cells were isolated from the lymphocyte fraction using the Regulatory T Cell Isolation kit (Miltenyi Biotec). T cells (1 × 10^6) were co-incubated in flat bottom 24-well plates with immature dendritic cells (1 × 10^5) and irradiated tumor cells (15,000 rad) (1 × 10^5) using AIM V medium supplemented with IL-2 (10 IU/ml), IL-10 (20 IU/ml), and IL-15 (20 IU/ml). Medium was exchanged on days 3 and 6. On day 9, the medium was replaced by fresh medium containing OKT-3 (1 μg/ml) and brefeldin A (1 μg/ml). 24 h later (day 10), lymphocytes and cell supernatants were separately harvested. Indomethacin (10 μg/ml) was added on days 0, 3, and 6 to some co-cultures. T cells were harvested from co-cultures as the non-adherent fraction, and their purity and viability routinely exceeded 95% (29). Tr1 generated in the presence of COX-2$^+$ tumor cells are designated as "Tr1/COX-2$^+$," whereas those generated in the presence of COX-2$^-$ tumor cells are designated as "Tr1/COX-2$^-$." Control or "reference cells" for IVA-generated Tr1 were CD4$^+$CD25$^-$ T cells cultured for 10 days in the absence of tumor cells or dendritic cells but in the presence of 150 IU/ml IL-2, which these T cells require for proliferation (28, 29).

Separation of Treg—Autologous CD4$^+$CD25^{high} T cells were single cell-sorted from CD4$^+$ T cell-enriched fractions of buffy coats obtained from the Central Blood Bank, Pittsburgh, PA using a Beckman Coulter cell sorter after staining the cells with the relevant antibodies as described previously (30).

Antibodies—The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-CD3-ECD, anti-CD4-ECD, anti-CD4-PC5, anti-CD25-PC5, anti-GITR-FITC, anti-FOXP3-FITC, anti-CD39-FITC, anti-CD39-PE, anti-CD73-PE, anti-COX-2-FITC, anti-CD122, anti-CD132, anti-IL-10, anti-TGF-β_1, and anti-CTLA-4-PE mAbs and their relevant isotypes, which served as negative controls for surface or intracellular staining. All were purchased from Beckman Coulter except for the following: anti-FOXP3, anti-CD39-FITC, and anti-CD39-PE, which were purchased from eBioscience; anti-CD73-PE, which was purchased from BD Pharmingen; anti-GITR-FITC, anti-CTLA-4-PE, anti-IL-10, and anti-TGF-β_1, which were purchased from R&D Systems; and anti-COX-2-FITC, which was purchased from Cayman Chemicals. Before use, all Abs were titrated using resting as well as activated PBMC obtained from NC to determine the optimal staining dilutions.
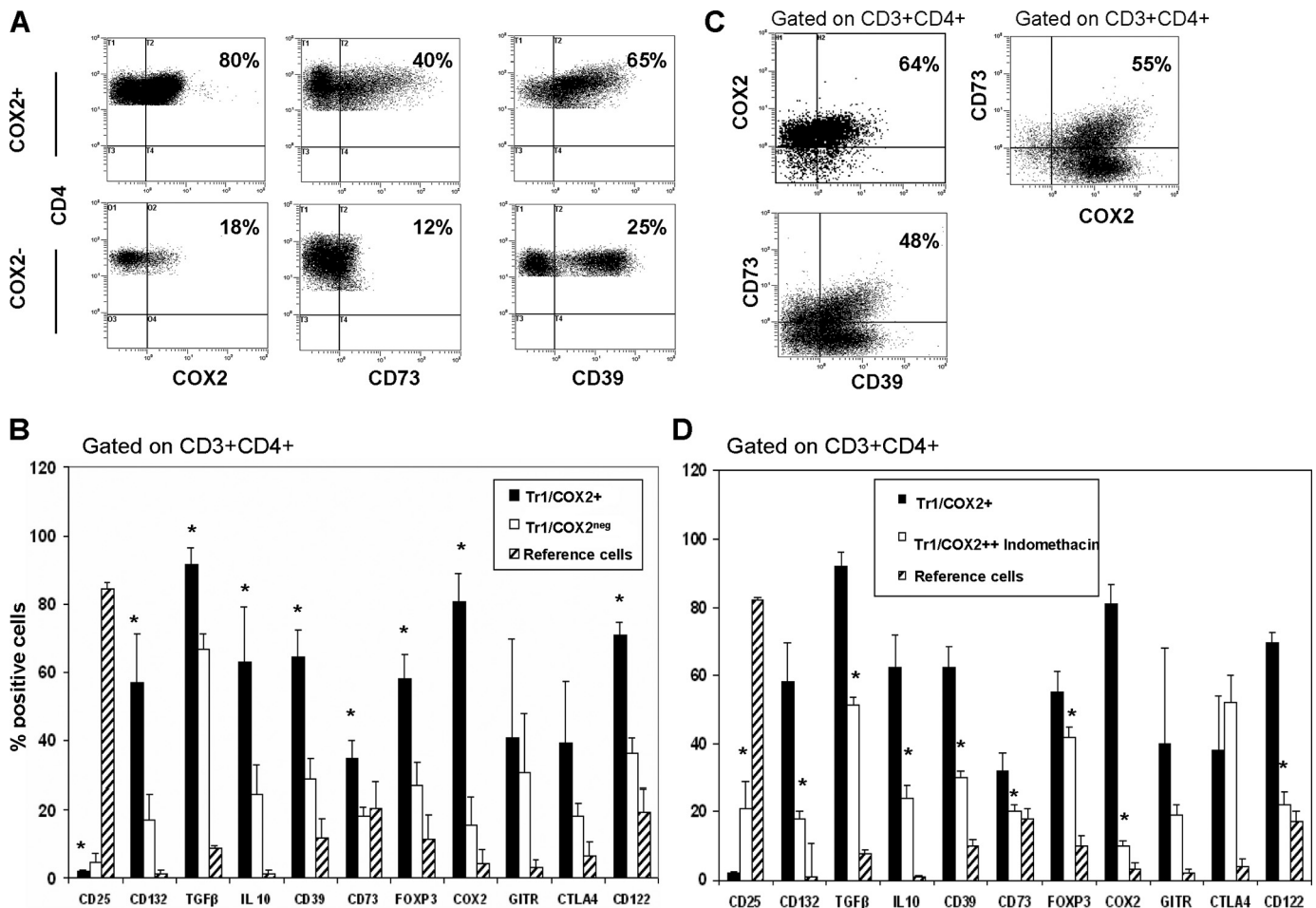
FIGURE 1. **Phenotypic characterization of IVA-generated Tr1.** *A*, representative fluorescence-activated cell sorting plots for marker expression in Tr1 cells generated in the presence of either COX-2$^+$ or COX-2$^-$ tumor cells. *B*, flow cytometry analysis of Tr1 cells generated in the presence of a COX-2$^+$ or COX-2$^-$ tumor cell line. CD4$^+$CD25$^+$ T cells cultured for 10 days in the presence of 150 IU/ml IL-2 but in the absence of tumor cells and dendritic cells served as reference cells for IVA co-cultures here and in all other experiments. *C*, representative fluorescence-activated cell sorting plots for co-expression of COX-2, CD39, and CD73 in Tr1 cells generated in IVA (Tr1/COX-2$^+$). *D*, phenotypic analysis of Tr1 generated in the presence of COX-2$^+$ tumor cells with or without indomethacin. Data represent 10 independent experiments. *Asterisks* indicate a significant difference at $p < 0.05$. *Bars* indicate ±S.D.

Surface and Intracellular Staining—Freshly isolated cells or *in vitro* activated cells were stained for flow cytometry as described previously (10). Briefly, cells were incubated with mAbs specific for surface markers for 30 min at 4 °C in the dark and then fixed with 2% (w/v) paraformaldehyde in PBS for 15 min. Afterward, cells were permeabilized with 0.1% (w/v) saponin and stained with mAbs specific for intracellular markers for 30 min at 4 °C in the dark. Cells were washed twice with 0.1% saponin in PBS, resuspended in a flow solution, and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

Flow Cytometry—Flow cytometry was performed using a EPICS® XL-MCL flow cytometer equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in forward and side scatter. The forward scatter and side scatter were set in a linear scale, and 10^6 cells were acquired for analysis, which was performed using the Coulter EXPO 32vl.2 analysis program. For additional analyses, gates were restricted to the CD3$^+$CD4$^+$ subset.

Suppression Assay—AutoMACS-sorted CD4$^+$CD25$^-$ RC were stained with 1.5 μM CFSE (Molecular Probes/Invitrogen)

and incubated at 37 °C for 15 min as described previously (10). CFSE-labeled autologous CD4$^+$CD25$^-$ (10^{5}cells/well) were incubated in wells of flat bottom 96-well plates at the suppressor (S)/RC ratios of 1:1, 2:1, 5:1, and 10:1. T cells harvested from the 10-day IVA as well as the T cells from the reference culture served as S cells. Using the same assay design, the following inhibitors or modifiers were added to RC 30 min before adding the S cells: ARL67165 (250 μM); α,β-methylene ADP (100 μM); rolipram (25 μM), a phosphodiesterase (PDE)$_4$ inhibitor; cilostamide (25 μM), a PDE$_3$ inhibitor; indomethacin (30 μM); AH23848 (25 μM), an EP$_4$ antagonist; and AH6809 (25 μM), an EP$_1$ and EP$_2$ antagonist, purchased from Sigma-Aldrich; ZM241385 (0.3 μM) and SC19220 (25 μM), an EP$_1$ antagonist, purchased from Tocris Bioscience; and (R_p)-8-Br-cAMPS (30 μM) purchased from Alexis Biochemicals. All inhibitors were initially titrated to determine their optimal concentrations that were not toxic for T cells. RC were stimulated with plate-bound OKT-3 (2 μg/ml) and soluble anti-CD28 mAb (2 μg/ml) (Miltenyi Biotec) in the presence of 150 IU/ml IL-2 for 5 days. S cells were considered to mediate suppression when they significantly inhibited proliferation of RC in co-cultures. All CFSE data were analyzed using the ModFit software provided by
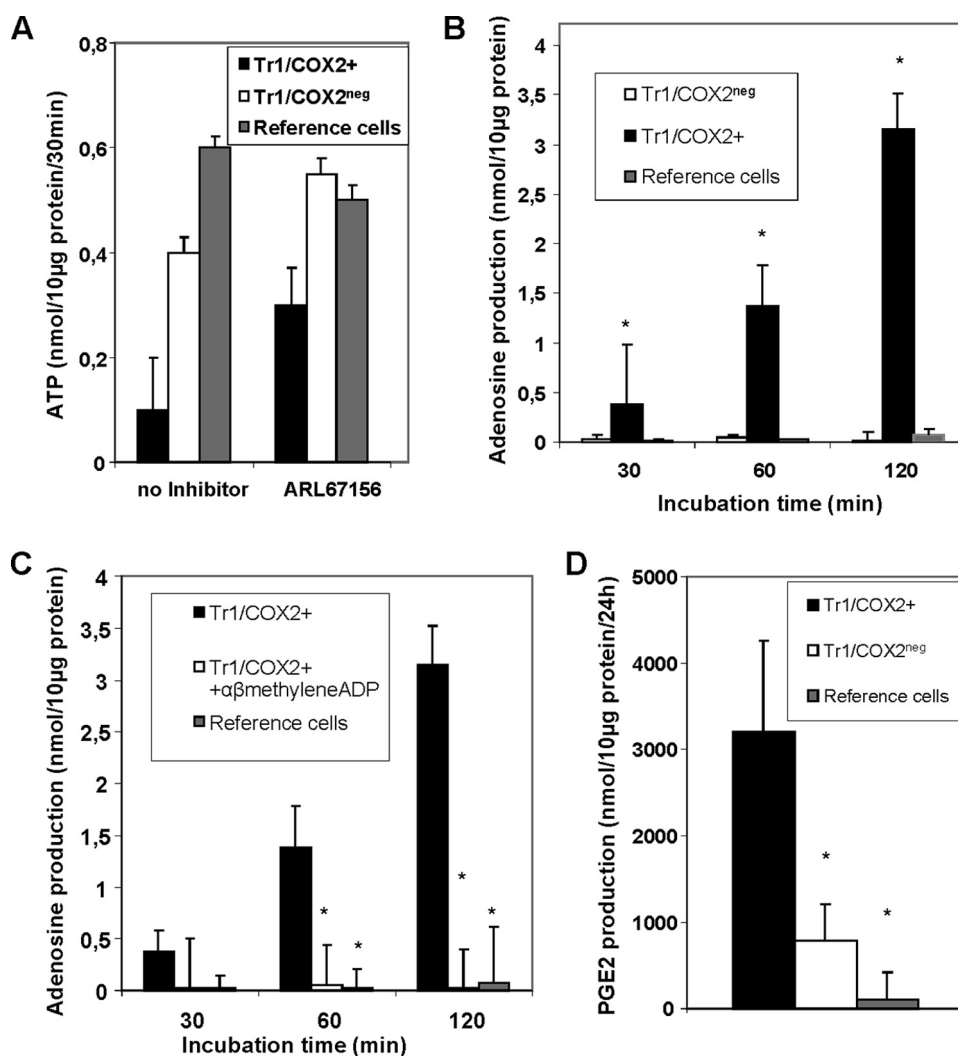
FIGURE 2. **Ectonucleotidase and COX-2 in Tr1.** *A*, hydrolysis of exogenous ATP is shown by CD39⁺ Tr1 cells generated in IVA that contained COX-2⁺ or COX-2⁻ tumor cells. In some experiments, ARL67156, a CD39 inhibitor, was added. *B*, adenosine production by Tr1 cells generated in the presence of COX-2⁺ or COX-2⁻ tumor cells was determined by mass spectrometry. *C*, in some experiments, α,β-methylene ADP, a CD73 inhibitor, was added. *D*, the production of PGE₂ by Tr1/COX-2⁺ or Tr1/COX-2⁻ was measured by ELISA. *A–D*, in each panel, data represent three independent experiments. *Asterisks* indicate significant differences at $p < 0.05$. *Bars* indicate $\pm$S.D.

Mass Spectrometric Analysis of Adenosine Production—T cells obtained from 10-day IVA or cells from the reference cultures were incubated with 10 μM exogenous ATP in wells of 96-well flat bottom plates. Cell supernatants were collected after various periods of incubation. Samples were boiled for 2 min and stored on dry ice until analysis. Adenosine was measured on a Thermo Finnigan LCQ Duo mass spectrometer equipped with electrospray ionization as described previously (4). The average concentration of adenosine was determined in duplicate wells.

ELISA and Enzyme Immunoassay—Media from Tr1 and control cultures were replaced on day 9 with fresh media containing OKT-3 (1 μg/ml) and anti-CD28 mAb (1 μg/ml), and cell supernatants were harvested after 24 h. Levels of PGE₂ were determined by ELISA (R&D Systems). cAMP levels were determined using the Amersham Biosciences cAMP Biotrak Enzyme Immunoassay System (GE Healthcare). Tr1 cells, nTreg, or RC were incubated overnight in 96-well round bottom plates. Cells were pretreated for 20 min with 2-chloroadenosine (5 μM; Sigma-Aldrich), PGE₂ (1 μM; Sigma-Aldrich), CGS21680 (2 μM; Sigma-Aldrich), butaprost (1 μM; Tocris Bioscience), rolipram (25 μM; Sigma-Aldrich), cilostamide (25 μM; Sigma-Aldrich), isobutylmethylxanthine (25 μM; Tocris Bioscience), or a combination of these reagents. All assays were performed according to the manufacturers' instructions.

Immunofluorescence—HNSCC tissue samples were embedded in OCT and 5 μM-thick frozen sections were cut in a cryostat, fixed for 10 min in cold acetone/ethanol (1:1), and dried at room temperature. The following anti-human Abs were used for staining: anti-CD4-FITC and anti-CD132-PE, both purchased from BD Pharmingen, and anti-CD39 and anti-COX-2, both purchased from Santa Cruz Biotechnology. As secondary Abs, Cy5-labeled donkey anti-rabbit and donkey anti-mouse (1:500; Jackson ImmunoResearch Laboratories) were used. To eliminate nonspecific binding of secondary Abs, tissue sections were first incubated with 10% donkey serum for 1 h and afterward washed extensively in PBS. Sections were incubated with the primary Abs for 1 h at room temperature in a moist chamber. Next, slides were washed and incubated with the secondary Abs under the same conditions. Primary Abs were omitted in all negative controls. Sections were

Verity Software House (Topsham, ME). The percentage of suppression was calculated by using the mean proliferation index of RC alone compared with the proliferation index of cultures containing RC + S cells. The program determines the percentage of cells within each detected peak; the sum of all peaks in the control culture is set to be 100% proliferation or 0% suppression.
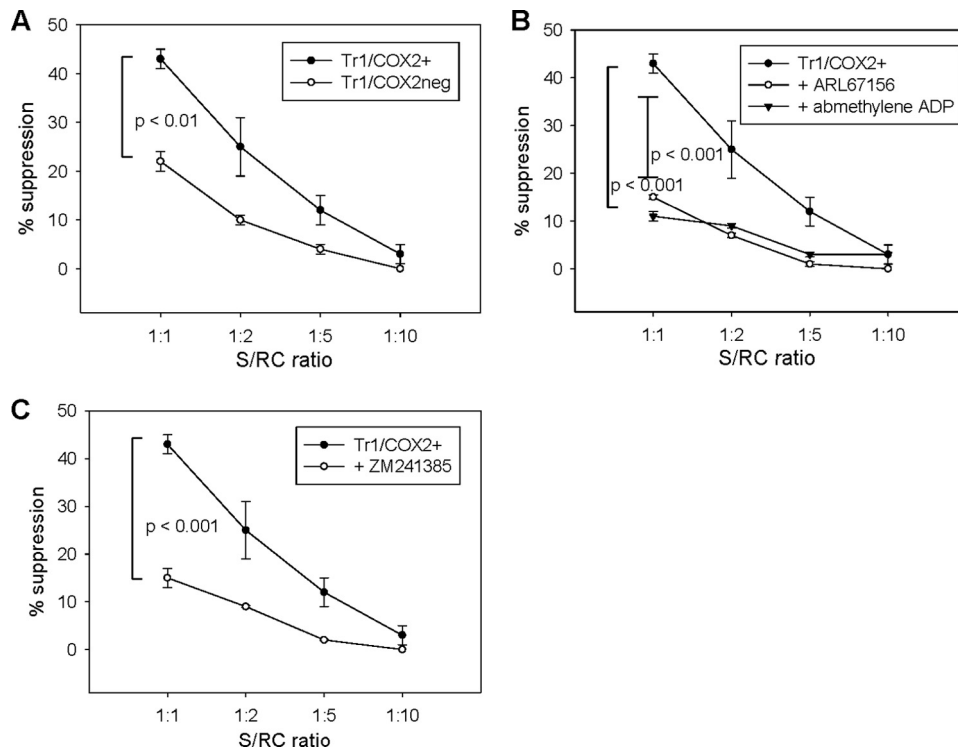
ATP Hydrolysis Assay—Tumor cells, T cells obtained from 10-day IVA, or cells from the reference cultures (25×10^3/well) were incubated in wells of flat bottom 96-well plates for 30 min with 10 μM ATP (Sigma-Aldrich). Cells in some wells were preincubated with ARL67156, an ectonucleotidase inhibitor, at a final concentration of 250 μM for 30 min prior to the addition of exogenous ATP. The concentration of "unhydrolyzed" ATP was determined in a luciferase-based detection system (ATP Lite Luminescence ATP Detection Assay System from PerkinElmer Life Sciences) as described previously (4). The average count was computed using triplicate wells.

FIGURE 3. **Suppression of RC proliferation mediated by Tr1/COX-2$^+$ and Tr1/COX-2$^-$ in presence and absence of various enzymatic inhibitors.** MACS-sorted CD4$^+$CD25$^-$ cells (RC) were CFSE-labeled and stimulated with plate-bound OKT-3 and soluble anti-CD28 in the presence of Tr1/COX-2$^+$ or Tr1/COX-2$^-$ and 150 IU/ml IL-2 for 5 days. Cells were analyzed by flow cytometry gating on CD4$^+$CFSE$^+$ T cell subsets and further analyzed using the ModFit program. *A*, suppression of CD4$^+$CD25$^-$ cell proliferation mediated by Tr1/COX-2$^+$ or Tr1/COX-2$^-$ at various S/RC ratios. *B* and *C*, to selected wells, ARL67156, a CD39 inhibitor; α,β-methylene ADP, a CD73 inhibitor; or ZM241385, a selective A$_{2a}$R and A$_{2b}$R antagonist, was added. Data are means $\pm$ S.D. from three individual experiments.

mounted in a mounting medium with DAPI (Vector Laboratories) to trace cell nuclei. Slides were evaluated under the Olympus Provis fluorescence microscope under ×400 magnification. For digital image analysis, the software Adobe Photoshop 7.0 version was used.

Statistical Analysis—All data are presented as means of at least three experiments ±1 S.D. The data were analyzed using the Student's *t* test, and *p* values <0.05 were considered to be significant.

RESULTS

Tr1 Generated in Presence of COX-2$^+$ or COX-2$^-$ Tumor Cells Have Distinct Phenotype—The phenotypic profiles of Tr1 generated in the IVA in the presence of COX-2$^+$ tumor cells (Tr1/COX-2$^+$) or COX-2$^-$ tumor cells (Tr1/COX-2$^-$) were evaluated by flow cytometry. Tumor cells were irradiated, and although they did not proliferate in co-cultures, they could have been a source of factors promoting Tr1 differentiation, *e.g.* PGE$_2$. Tr1/COX-2$^+$ cultures contained a significantly higher frequency (*p* < 0.01) of CD4$^+$CD39$^+$ and CD4$^+$CD73$^+$ T cells than did Tr1/COX-2$^-$ cultures (Fig. 1, *A* and *B*). The expression levels (mean fluorescence intensity) of CD39 and CD73 on Tr1 cells were also significantly higher in Tr1/COX-2$^+$ than Tr1/COX-2$^-$ populations (data not shown). In agreement with our previously reported data, the IVA cultures were enriched in CD4$^+$ T cells expressing the "conventional" Tr1 markers CD122, CD132, TGF-β_1, and IL-10 (28), and this enrichment

was significantly greater (*p* < 0.05) in the IVA generated in the presence of COX-2$^+$ than in the presence of COX-2$^-$ tumor cells (Fig. 1*B*). Interestingly, most of CD4$^+$ Tr1 cells co-expressed COX-2, CD73, and CD39 (Fig. 1*C*). In aggregate, these results suggested that PGE$_2$ derived from COX-2$^+$ tumor cells promotes the induction of Tr1 in the IVA as also reported by others (31, 32). To confirm this hypothesis, indomethacin, a COX-1 and COX-2 inhibitor, was added to the co-cultures containing COX-2$^+$ tumor cells. As expected, indomethacin significantly down-regulated ectonucleotidase and other Tr1 marker expression in Tr1 (Fig. 1*D*).

Ectonucleotidase Activity in Generated Tr1 Cells—Because CD39 is an ectonucleotidase responsible for ATP hydrolysis, we wished to determine whether the IVA-generated CD39$^+$ Tr1 cells were functionally active by incubating them with 10 μM exogenous ATP for 30 min. Compared with Tr1/COX-2$^-$ or control cells, Tr1/COX-2$^+$ hydrolyzed more ATP (Fig. 2*A*; *p* < 0.05). Upon the prior addition of ARL67156, a selective inhibitor of ecto-ATPases, the ability of these cells to hydrolyze ATP was decreased (Fig. 2*A*; *p* < 0.05).

Adenosine Production by Tr1—We next asked whether CD39 and CD73 expression endowed Tr1 cells with the ability to produce adenosine following the addition of 10 μM exogenous ATP. Adenosine levels were measured in Tr1/COX-2$^+$, Tr1/COX-2$^-$, and reference cell supernatants collected at various time points after ATP addition. As shown in Fig. 2*B*, Tr1/COX-2$^+$ produced more adenosine than Tr1/COX-2$^-$ or reference cells (*p* < 0.05). Upon co-incubation of Tr1/COX-2$^+$ with α,β-methylene ADP, a specific CD73 inhibitor, adenosine production was almost completely inhibited (Fig. 2*C*; *p* < 0.02).

PGE$_2$ Production by Tr1—As most of the generated Tr1 expressed COX-2 (Fig. 1*C*), we measured their ability to also produce PGE$_2$. Supernatants of Tr1/COX-2$^+$ and Tr1/COX-2$^-$ cultures were collected after 24-h stimulation with OKT-3 and anti-CD28 mAb, and levels of PGE$_2$ were measured by ELISA. As shown in Fig. 2*D*, Tr1/COX-2$^+$ produced significantly higher levels of PGE$_2$ than Tr1/COX-2$^-$ or reference cells (*p* < 0.004).

Suppressor Function of Tr1—To analyze suppressor activity of Tr1/COX-2$^+$ and Tr1/COX-2$^-$, the cells were harvested from the IVA and co-incubated with autologous CD4$^+$CD25$^-$ RC at different S/RC ratios. After a 5-day culture, the mean suppressor activity of Tr1/COX-2$^+$ at the 1:1 S/RC ratio was higher than that of Tr1/COX-2$^-$ (Fig. 3*A*; *p* < 0.01). The suppression of proliferation linearly decreased

upon further dilution of S (Fig. 3*A*). Cells from control cultures did not suppress proliferation of RC (data not shown). These results indicate that the immunosuppressive activity of Tr1 was related to expression of ectonucleotidases and COX-2 in these cells.

Adenosine Is Involved in Tr1-mediated Suppression—Because adenosine is known to mediate immunosuppression by engaging cell surface receptors (A$_{2A}$R) on effector T cells, we next used ARL67156, a structural analogue of ATP and an ectonucleotidase inhibitor, to confirm that adenosine generated by Tr1 is responsible for suppression of RC proliferation. Upon addition of ARL67156 to selected co-cultures, a significant decrease in suppression was observed compared with cultures without the inhibitor (Fig. 3*B*, $p < 0.001$). In the same type of co-cultures, the addition of α,β-methylene ADP, an inhibitor of CD73, also resulted in reduction of Treg-mediated suppression (Fig. 3*B*; $p < 0.001$).

Because the immunosuppressive effects of adenosine are expected to be mediated via the A$_{2A}$R expressed on effector T cells, we blocked this receptor on RC in the co-culture by adding ZM241385, a selective A$_{2A}$R and A$_{2B}$R antagonist, to selected wells. The addition of ZM241385 significantly reduced suppression mediated by Tr1/COX-2$^+$ at the 1:1 S/RC ratio (Fig. 3*C*; $p < 0.001$).

PGE$_2$ Has an Additive Effect with Adenosine in Tr1-mediated Suppression—Having determined that Tr1 express COX-2 and produce PGE$_2$, we next asked whether PGE$_2$ contributed to Tr1-mediated suppression. Adding indomethacin, a non-selective COX-1 and COX-2 inhibitor, to selected co-culture wells decreased suppression mediated by Tr1 (Fig. 4*A*; $p < 0.05$). An even further decrease in Tr1-mediated suppression was observed when both indomethacin and α,β-methylene ADP were added to selected wells (Fig. 4*A*; $p < 0.05$). As PGE$_2$ mediates its biological effects by binding to four distinct surface receptors, namely EP$_1$, EP$_2$, EP$_3$, and EP$_4$, we next determined which receptors are predominantly involved in the observed suppression of RC proliferation. As shown in Fig. 4*B*, the addition of AH6809, an EP$_2$R antagonist, to co-cultures caused a significant decrease in suppression of RC proliferation, whereas AH23848, an EP$_4$R antagonist, and all other EPR antagonists had no effect on RC proliferation (data not shown) as also noted by Mahic *et al.* (18). The data suggest that signaling via the EP$_2$R is largely responsible for PGE$_2$-mediated suppression of RC proliferation by Tr1 cells generated in the presence of COX-2$^+$ tumor cells. A further decrease of Tr1-mediated suppression was observed when both AH6809 and ZM241385 were added to selected culture wells (Fig. 4*B*).

cAMP Production in nTreg, Tr1, and RC—Signaling via the A$_{2a}$R and EP$_2$R is expected to lead to activation of adenylate cyclase in RC and to increased production of cytosolic cAMP. To evaluate the intracellular signaling pathway involved in Tr1-mediated suppression, we incubated RC, Tr1, and nTreg (CD4$^+$CD39$^+$CD25^{high}) subsets in the presence of various cAMP inducers and then measured intracellular cAMP concentrations. As shown in Fig. 5*A*, base-line cAMP levels were higher in nTreg than in RC. Chloroadenosine (CADO) as well as PGE$_2$ increased cAMP levels only in RC with an additive effect when used in combination.
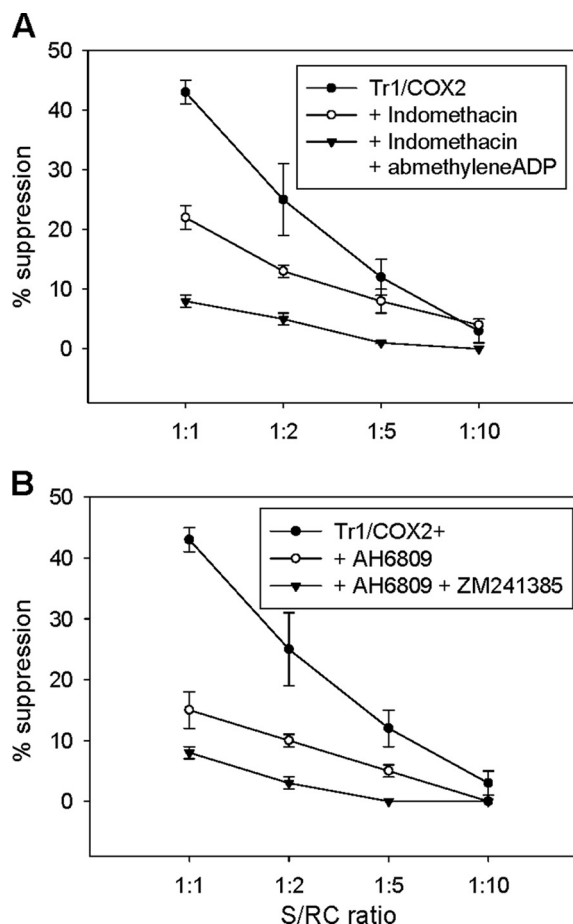
FIGURE 4. **Suppression of RC proliferation mediated by Tr1/COX-2$^+$ in presence and absence of various adenosine and prostaglandin receptor antagonists.** MACS-sorted CD4$^+$CD25$^-$ cells (RC) were CFSE-labeled, stimulated, and cultured as described in the legend to Fig. 3. *A* and *B*, suppression of CD4$^+$CD25$^-$ cell proliferation mediated by Tr1 cells at various S/RC ratios in the absence or presence of indomethacin, a nonselective COX-1 and COX-2 inhibitor; α,β-methylene ADP, a CD73 inhibitor; AH6809, an EP$_2$R antagonist; ZM241385, a selective A$_{2A}$R and A$_{2B}$R antagonist; or a combination. Data are means ± S.D. from three independent experiments.

The EP$_2$R agonist butaprost and the A$_{2A}$R agonist CGS21680 similarly increased cAMP levels only in RC. The intracellular levels of cAMP are balanced by its production and hydrolysis through PDEs. As it remains unclear whether Tr1 and RC express the same or different PDE subtypes, we tested various inhibitors of PDE subtypes for their ability to inhibit cAMP hydrolysis in nTreg, Tr1, and RC. As shown in Fig. 5*B*, cAMP concentrations were increased upon stimulation with chloroadenosine only in RC but not in nTreg or Tr1 after preincubation of the cells with rolipram, a specific PDE$_4$ inhibitor. However, upon preincubation of these cell subsets with cilostamide, a specific PDE$_3$ inhibitor, significant up-regulation of cAMP production was evident in nTreg and Tr1 but not in RC. Isobutylmethylxanthine, a nonspecific inhibitor of all PDEs, increased cAMP levels in all tested cell subsets similarly to a combination of cilostamide and rolipram. These data suggest that the hydrolysis of cAMP in RC is primarily controlled by PDE$_4$, whereas in Treg, it is controlled by PDE$_3$ (Fig. 5).

Inhibition of cAMP Pathway and Tr1-mediated Immune Suppression—The inhibition of PDE is expected to result in an increase of intracellular cAMP levels/activity in RC and
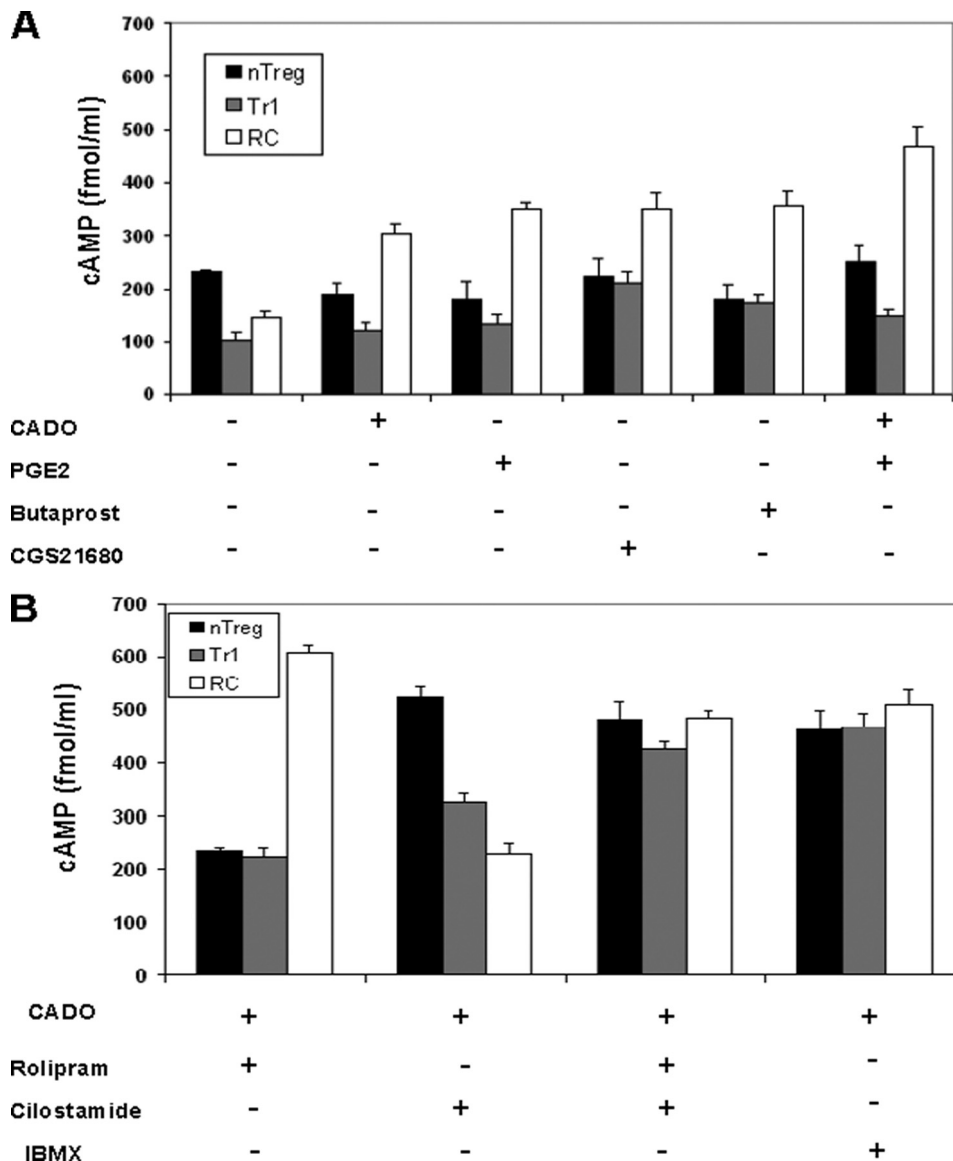
FIGURE 5. **Modulation of intracellular cAMP levels in different T cell subsets.** IVA-generated Tr1/COX-2$^+$, nTreg, or RC (100,000 cells/well) were incubated in 96-well plates in the absence or presence of various reagents. Intracellular cAMP levels were measured by enzyme immunoassay after lysing the cells. Data are means ± S.D. from three independent experiments. *IBMX*, isobutylmethylxanthine; *CADO*, chloroadenosine.

in an inhibition of Treg-mediated suppression of RC proliferation.

Different Tr1 Subsets in Peripheral Blood of HNSCC Patients—Our *in vitro* data have shown that PGE$_2$ and adenosine play a major role in suppression mediated by Tr1. To confirm the involvement of these molecules in cancer-related suppression, PBMC of patients with HNSCC were evaluated for the presence of Tr1 subsets, including IL-10$^+$ and TGF-$\beta_1$$^+$ subpopulations. Furthermore, the frequency of Tr1 in the blood of these patients was compared prior to and after oncological treatment, and it was compared in the blood of normal controls. As also described previously (33), an increase in CD39$^+$ and COX-2$^+$ Treg was observed among CD4$^+$ T cells in the peripheral blood of HNSCC patients. A further increase occurred after oncological therapy. IL-10$^+$ and TGF-β^+ CD4$^+$ T cells were also elevated in patients with HNSCC, but the frequency of these cells was significantly reduced after therapy (Fig. 7*A*). CD39, CD73, and COX-2 appear to be co-expressed in CD4$^+$ T cells, supporting the hypothesis that adenosinergic and COX-2-mediated suppressive pathways are used in concert by Tr1 (Fig. 7*B*). However, no overlap of these cells with either the CD4$^+$TGF-β^+ or CD4$^+$IL-10$^+$ subsets was evident (Fig. 7*B*). Furthermore, CD4$^+$COX-2$^+$, CD4$^+$CD39$^+$, and CD4$^+$CD73$^+$ cells co-expressing FOXP3 represented 54 ± 12, 58 ± 8, and 56 ± 11%, respectively, and those co-expressing CTLA-4 were 39 ± 15, 45 ± 11, and 41 ± 8%, respectively, in the gate set on CD4$^+$ T cells (data not shown).

Co-expression of CD39 and COX-2 in Tr1 Present in Tumor—As we observed co-expression of CD39 and COX-2 in circulating Tr1 in HNSCC patients, we asked whether tumor-infiltrating Tr1 also expressed these markers. HNSCC tissues were stained with the relevant mAbs and analyzed using multicolor immunofluorescence and confocal microscopy. We observed expression of CD39 and COX-2 in Tr1 cells with the majority of these cells showing positivity for both markers (Fig. 7*C*, *violet*; see *inset*). Effector T cells were negative for both markers (Fig. 7*C*, *green*). These findings support the conclusion that Tr1 cells in cancer patients co-express CD39 and COX-2 and, therefore, can produce both immunosuppressive adenosine and PGE$_2$.

increased RC sensitivity to Treg-mediated suppression. When rolipram, a specific inhibitor of PDE$_4$, was added to co-cultures of RC with Treg, it caused a significant up-regulation of nTreg- or Tr1-mediated suppression (Fig. 6, *A* and *B*). In contrast, cilostamide, a specific PDE$_3$ inhibitor, showed no effects on Treg-mediated suppression (data not shown). These findings suggest that binding of Treg-derived PGE$_2$ to EP$_2$R on RC mediates suppression by increasing cAMP levels in these cells.
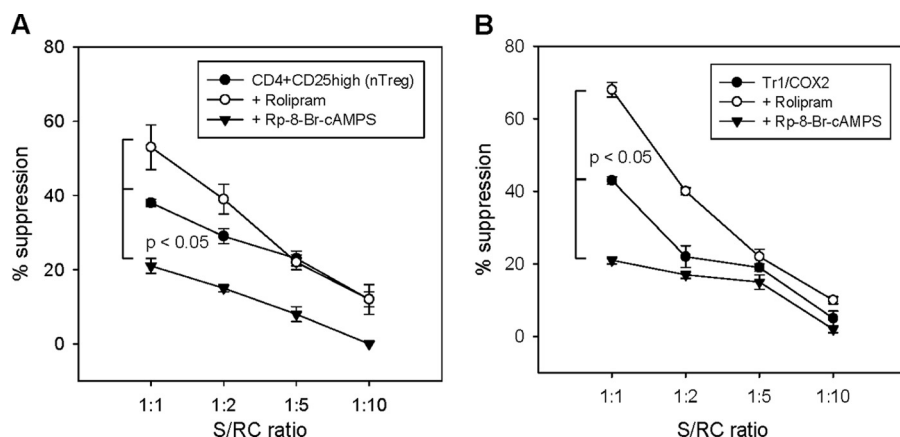
We also tested whether blocking of PKA type I activity in RC could inhibit suppression by nTreg or Tr1. To this end, (R_p)-8-Br-cAMPS, an inhibitor of the regulatory subunit of the cAMP-dependent PKA that preferentially inhibits PKA type I, was added to the co-cultures of RC with Treg. A significant decrease ($p < 0.05$) of suppression of RC proliferation mediated by nTreg or Tr1 was observed (Fig. 6). The data suggest that blocking of PKA type I activity in RC results

FIGURE 6. **Impact of intracellular cAMP signaling on Tr1- or nTreg-mediated suppression.** MACS-sorted CD4$^+$CD25$^-$ cells (RC) were CFSE-labeled, stimulated, and cultured as described in the legend to Fig. 3. *A* and *B*, suppression of CD4$^+$CD25$^-$ cell (RC) proliferation mediated by Tr1/COX-2$^+$or CD25^{high} nTreg cell subsets at various S/RC ratios in the absence or presence of rolipram, a PDE$_4$ inhibitor, or (R_p)-8-Br-cAMPS, an inhibitor of the regulatory subunit of the cAMP-dependent PKA. The inhibitors were added to the Tr1 co-cultures on days 0, 3, 6, and 9. Data are means ± S.D. from three independent experiments.

DISCUSSION

In this study, we demonstrate for the first time that human inducible Treg or Tr1, like nTreg, express ectonucleotidases and are able to generate immunosuppressive adenosine. The Tr1 generated in IVA also expressed COX-2 and produced PGE$_2$, a well known suppressor of T cell proliferation. In blocking experiments with inhibitors, it was possible to show that adenosine and PGE$_2$ participate in Tr1-mediated suppression of autologous RC proliferation. This is consistent with our previous report showing that treatment with adenosine and PGE$_2$ cooperatively diminishes lympho-
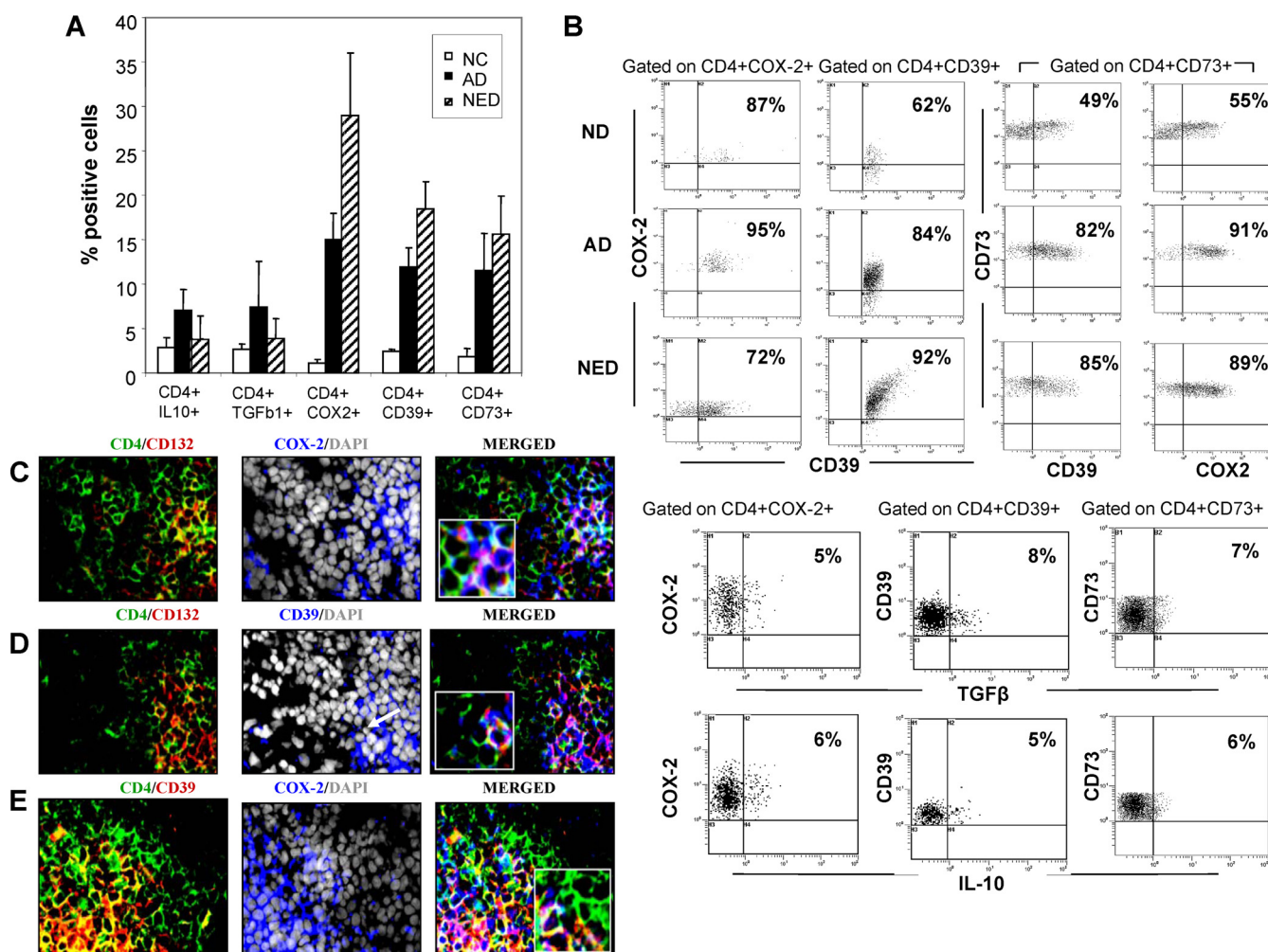
FIGURE 7. **Phenotypic analysis of different Tr1 subsets in peripheral blood and tumor tissues of HNSCC patients.** *A*, PBMC from NC and patients with AD or NED were isolated from whole blood and stained for different Tr1 markers. Cells were analyzed by flow cytometry, gating on the CD3$^+$CD4$^+$ subset. Data represent five individuals in each cohort. *Asterisks* indicate a significant difference at $p < 0.05$. *B*, representative dot blots for NC, AD, and NED show COX-2, CD39, and CD73 expression by flow cytometry and co-expression with IL-10 and TGFβ_1. *C*, *D*, and *E*, CD4$^+$CD132$^+$COX-2$^+$, CD4$^+$CD132$^+$CD39$^+$, and CD4$^+$CD39$^+$COX-2$^+$ T cells shown in sections of a representative tumor of five examined (*magnification*, ×400; *inset magnification*, ×800). *C*, sections stained for CD4, CD132, and COX-2. CD4$^+$ cells are *green*, CD132$^+$ cells are *red*, CD4$^+$CD132$^+$ cells are *yellow*, and CD4$^+$CD132$^+$COX-2$^+$ cells are *violet*. *D*, sections stained for CD4, CD132, and CD39. CD4$^+$ cells appear in *green*, CD132$^+$ cells are *red*, CD4$^+$CD39$^+$ cells are *yellow*, and CD4$^+$CD132$^+$CD39$^+$ cells are *violet*. *E*, sections stained for CD4, CD39, and COX-2. CD4$^+$ cells are visible in *green*, CD39$^+$ cells are *red*, CD4$^+$CD39$^+$ cells are *yellow*, and CD4$^+$CD39$^+$COX-2$^+$ cells are *violet*. Co-expression of CD39 and COX-2 on Tr1 cells *in situ* is observed.

cyte activity (34). CD39, CD73, and PGE_2 are co-expressed not only in cultured Tr1 but also in Tr1 present in the circulation and at tumor sites of patients with HNSCC. Their combined effects appear to be mediated through intracellular cAMP signaling. In contrast to adenosine- and PGE_2-producing Tr1, those Tr1 that express IL-10 and TGF-β seem to belong to a distinct subset of inducible Treg, emphasizing the diversity of mechanisms responsible for Tr1-mediated suppression.

The common intracellular cAMP pathway that inducible Treg utilize to mediate suppression is activated after adenosine, PGE_2, or both bind to their respective receptors expressed on RC. Tr1 cells are a source of both these mediators. Adenosine as well as PGE_2 suppresses RC activity via G-protein-coupled purinergic receptors present on the RC cell surface (34). We showed that $A_{2a}R$ and EP_2R on RC are predominantly involved in mediating suppression, which leads to a profound increase of intracellular cAMP in RC. Although the EP_4R is also coupled to the cAMP pathway, it seems to play only a minor part in these interactions possibly because, in comparison with EP_2R, EP_4R is the low affinity receptor or because the EP_4R is internalized rapidly after PGE_2 binding (22, 35).

In regard to the regulation of intracellular cAMP levels, we show that a selective inhibition of PDE_4 by rolipram in RC increases cAMP levels and potentiates suppression of RC proliferation by Treg. Interestingly, in contrast to RC, human Tr1 and nTreg seem to utilize PDE_3 for cAMP hydrolysis as has been reported previously in the mouse by Rudensky and co-workers (36). This preferential use of PDE_3 by Tr1 further exemplifies the diversity of signals that regulate the common cAMP pathway in human lymphocyte subsets. Furthermore, neither PDE_1 nor PDE_2 seem to be involved in intracellular cAMP hydrolysis in these cell subtypes because simultaneous inhibition of PDE_3 and PDE_4 did not alter intracellular cAMP levels. It has also been reported that PKA type I, rather than PKA type II, is involved in cAMP-mediated immune suppression (37, 38). Our current data support this hypothesis, and we have shown that Tr1-mediated suppression can be substantially abrogated by PKA type.

We have reported previously and further confirm here that a subset of Tr1 cells can mediate suppression through TGF-β_1 and IL-10 secretion (39). Because of the possibility that distinct subsets of Tr1 exist and utilize different mechanisms of suppression in *vivo*, it was important to identify Tr1 that are predominantly engaged in suppression of immune reactivity in patients with cancer. As reported previously by us, the Tr1 frequency was found to be increased in HNSCC patients compared with NC and was higher in those with NED than in patients with AD (33). We have also observed that after oncological treatment the $CD39^+$ as well as $COX-2^+$ Tr1 subsets increased in frequency, whereas the proportions of TGF-β_1^+ and IL-10 Tr1 significantly decreased. A larger proportion of $CD4^+$ cells expressed CD39 and COX-2 in NED patients. Although these cross-sectional data were obtained with small cohorts of patients, they confirm our previously reported results (33). A possible explanation for these findings could be that chronic inflammation resulting from oncological therapies leads to constant ATP release into tissue due to cell death. This ATP release up-regulates CD39 and COX-2 expression in Tr1,

leading to greater production and release of adenosine and PGE_2. In fact, up-regulation of these molecules is known to occur during inflammation (40, 41). On the other hand, the two cytokines IL-10 and TGF-β are inter-related in Treg and positively regulate each other's generation (42). The role of IL-10 in Treg in cancer seems to be controversial. It has been proposed that although IL-10 plays a role in Treg-mediated immune suppression it also sensitizes Treg to apoptosis (43). Furthermore, it has been reported that Treg prevent rather than enhance cancer progression by IL-10 production (44), which could explain its decreased expression in Treg of patients with NED. Tumor microenvironments are characterized by the secretion of many molecules that promote tumor progression utilizing distinct mechanisms. These molecules regulate and determine the phenotype and functional potential of immune cells in the microenvironment, including Tr1. Thus, the tumor microenvironment enriched in adenosine and PGE_2 favors the expansion of more immunosuppressive $COX-2^+$ and ectonucleotidase$^+$ T cells.

In summary, this study demonstrates for the first time the co-expression of CD39, CD73, and COX-2 in Tr1 cells that are adenosine and PGE_2 producers and thus can mediate high levels of suppression. The mechanism of suppression involves binding of adenosine and PGE_2 to $A_{2a}R$ and EP_2R, respectively, on RC and increasing intracellular cAMP levels. This subset of Tr1 is enriched in the tumor and peripheral circulation of cancer patients. Inhibition of PGE_2/adenosine-mediated suppression by Tr1, perhaps at the level of a common intracellular signaling pathway, might be a new approach for improving therapy for patients with cancer.

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